



Pulmonary, gastrointestinal and urogenital pharmacology

Leukotriene C₄ induces bronchoconstriction and airway vascular hyperpermeability via the cysteinyl leukotriene receptor 2 in S-hexyl glutathione-treated guinea pigs

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ABSTRACT

Cysteinyl leukotrienes act through G-protein-coupled receptors termed cysteinyl leukotriene 1 (CysLT₁) and cysteinyl leukotriene 2 (CysLT₂) receptors. However, little is known about the pathophysiological role of CysLT₂ receptors in asthma. To elucidate the possible involvement of CysLT₂ receptors in bronchoconstriction and airway vascular hyperpermeability, we have established a novel guinea pig model of asthma. *In vitro* study confirmed that CHO-K1 cells, expressing guinea pig CysLT₂ and CysLT₁ receptors are selectively stimulated by LTC₄ and LTD₄, respectively. However, when LTC₄ was intravenously injected to guinea pigs, the resulting bronchoconstriction was fully abrogated by montelukast, a CysLT₁ receptor antagonist, indicating rapid metabolism of LTC₄ to LTD₄ in the lung. We found that treatment with S-hexyl glutathione (S-hexyl GSH), an inhibitor of gamma-glutamyl transpeptidase, significantly increased LTC₄ content and LTC₄/(LTD₄ plus LTE₄) ratio in the lung. Under these circumstances, LTC₄-induced bronchoconstriction became resistant to montelukast, but sensitive to Compound A, a CysLT₂ receptor antagonist, depending on the dose of S-hexyl GSH. Combination with montelukast and Compound A completely abrogated this spasmogenic response. Additionally, we confirmed that LTC₄ elicits airway vascular hyperpermeability via CysLT₂ receptors in the presence of high dose of S-hexyl GSH as evidenced by complete inhibition of LTC₄-induced hyperpermeability by Compound A, but not montelukast. These results suggest that CysLT₂ receptors mediate bronchoconstriction and airway vascular hyperpermeability in guinea pigs and that the animal model used in this study may be useful to elucidate the functional role of CysLT₂ receptors in various diseases, including asthma.

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1. Introduction

Cysteinyl leukotrienes (CysLTs, including LTC₄, LTD₄ and LTE₄) play an important role in both the bronchoconstriction and airway inflammation associated with asthma. Recent advances in molecular biology have shown that CysLTs act via two G protein-coupled receptors known as cysteinyl leukotriene 1 (CysLT₁) (Lynch et al., 1999; Sarau et al., 1999) and cysteinyl leukotriene 2 (CysLT₂) receptors (Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000) and that both LTD₄ and LTC₄ equally bind to the human CysLT₂ receptor (Heise et al., 2000). Like the CysLT₁ receptor, the CysLT₂ receptor is expressed on airway smooth muscle cells (Heise et al., 2000), inflammatory cells (Figuerola et al., 2003; Mita et al., 2001; Mellor et al., 2003; Gauvreau

et al., 2000) and vascular endothelial cells (Sjöström et al., 2003), suggesting that this receptor may also be involved in asthma. However, studies showing that a CysLT₁ receptor antagonist almost completely inhibits LTD₄- or LTC₄-induced bronchoconstriction in guinea pig (Nakagawa et al., 1992; Muraki et al., 2009) undermine the physiological importance of CysLT₂ receptors in asthma.

On the other hand, cDNA encoding the guinea pig CysLT₂ receptor has been cloned (Ito et al., 2008), and agonist-stimulated signal transduction has clearly shown species difference in the reactivity of CysLT₂ receptor natural ligands between human and guinea pig. Whereas LTD₄ is a potent ligand not only for the CysLT₁ receptor, but also for the CysLT₂ receptor in humans, it is much less potent than LTC₄ for the guinea pig-CysLT₂ receptor (Ito et al., 2008). Since LTC₄ is rapidly transformed to LTD₄ by metabolizing enzymes, such as gamma-glutamyl transpeptidase (γ-GTP) (Orning and Hammarström, 1980; Snyder et al., 1984) and gamma-glutamyl leukotrienase (Lieberman et al., 1999; Han et al., 2002) in the body, it would be very difficult to induce CysLT₂ receptor-mediated biological responses

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in guinea pigs. Such species difference may hamper studies on the role of CysLT₂ receptors in asthma and other diseases.

To induce CysLT₂ receptor-mediated biological response in guinea pigs, we hypothesized that it is necessary to block LTC₄ metabolism to LTD₄, thereby maintaining enough LTC₄ at the target organ. In our experiment, we focused on S-hexyl glutathione (S-hexyl GSH), a known synthetic substrate of γ -GTP (Bäck et al., 2001) and assumed that this substrate can be metabolized in place of LTC₄ since, like LTC₄, it has an γ -glutamyl-L-cysteinyl-glycine moiety (Bäck, 2002). Using S-hexyl GSH, we investigated in this study conditions under which CysLT₂ receptor-mediated bronchial response might be brought about in guinea pigs. Through the experiments conducted in this study, we succeeded in developing a guinea pig model of asthma mediated by CysLT₂ receptors alone or by both CysLT₂ and CysLT₁ receptors, depending on the dose of S-hexyl GSH. Our results clearly elucidate the contribution of CysLT₂ receptors in asthma-associated bronchoconstriction and airway vascular hyperpermeability *in vivo*.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Japan SLC, Shizuoka, Japan) aged 6–7 weeks were used in this study. They were housed in an air-conditioned room maintained at 24 ± 2 °C and $55 \pm 5\%$ relative humidity with alternating 12 h light/dark cycles and provided with food (LRC4, Oriental Yeast Co., Ltd., Japan) and tap water *ad libitum*. All animal experiments were approved by the Animal Ethical Committee of Ono pharmaceutical Co., Ltd. and performed in accordance with the institutional animal care guidelines.

2.2. Drugs and materials

Montelukast was obtained from Sequoia Research Products, Ltd. (Pangbourne, United Kingdom). Compound A; 1-(5-carboxy-2-{3-[4-(3-cyclohexylpropoxy)phenyl] propoxy}benzoyl)-4-piperidinecarboxylic acid was synthesized in our laboratories and used as CysLT₂ receptor antagonist. Others materials used in this study were purchased commercially: LTC₄, LTD₄, LTE₄, LTC₄-d5, LTD₄-d5, and LTE₄-d5 (Cayman Chemical Company, MI, USA), S-hexylglutathione and fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) (Sigma-Aldrich, MO, USA). Montelukast was suspended in 0.5 w/v% methylcellulose solution and orally administered 24 h before LTC₄ challenge. Compound A was dissolved in physiological saline containing 1 vol% WellSolve (Celeste Corporation, Tokyo, Japan) and intravenously administered approximately 1 min before, or immediately before, LTC₄ challenge.

2.3. Preparation of cells stably expressing guinea pig CysLT₁ and CysLT₂ receptors

Full-length DNA encoding guinea pig CysLT₁ receptor was chemically synthesized. Kozak consensus sequence and N-terminal hemagglutinin epitope tag sequence were artificially introduced to the vector. These fragments were subcloned into mammalian expression vector, pEF1 (Life Technologies). Full-length DNA encoding guinea pig CysLT₂ receptor was amplified from guinea pig heart mRNA by RT-PCR. Kozak sequence and N-terminal myc epitope tag sequence were artificially introduced to the vector. These fragments were subcloned into mammalian expression vector, pIRES (Clontech Laboratories). The receptor sequences were verified by sequencing and confirmed identity with GenBank accession number AY236968 or AY236969. Both constructs were transfected with lipofectamine (Life Technologies) into CHO-K1 cells. After selection with geneticin, positive clones

obtained by the limited dilution technique were identified by stimulation with LTD₄ or LTC₄.

2.4. Calcium response assay

Intracellular calcium response was measured using Fura 2-AM (Dojindo) according to the manufacture's protocol. Briefly, the CHO-K1 cells stably expressing guinea pig CysLT₁ and CysLT₂ receptors were cultured in 96 well plates (Corning Incorporated) for at least 24 h (3×10^4 cells/well). The culture medium (Ham's F-12 supplemented with 10% fetal bovine serum and 500 μ g/ml geneticin) was removed, and the loading medium containing 5 μ M Fura 2-AM was added to each well. After 1 h incubation, the loading medium was removed, and the cells were rinsed with the assay buffer (Hanks' Balanced Salt Solution containing 20 mM HEPES). The assay buffer was then added to each well, and the cells were further incubated at room temperature for 1 h. The cells were next alternately irradiated at two excitation wavelengths (340 and 380 nm) using FDSS-3000 (Hamamatsu Photonics Co., Ltd., Shizuoka, Japan) to measure the ratio of fluorescence intensity (f_{340}/f_{380}) at 500 nm. Receptors natural ligands, i.e. LTC₄, LTD₄ and LTE₄ (0.001 nM to 1 μ M), were each added 30 s after commencement of the measurement of fluorescence intensity. In an experiment to evaluate Compound A antagonistic activity for CysLT₁ and CysLT₂, montelukast or Compound A was added 30 min before addition of the ligand (10 nM LTD₄ for the CysLT₁ receptor or 3 nM LTC₄ for the CysLT₂ receptor).

2.5. Preparation of lung tissue samples and quantification of CysLTs

Guinea pigs were anesthetized with pentobarbital sodium (75 mg/kg, i.p., Somnopentyl[®]), and a polyethylene cannula was inserted into the trachea. The other end of the cannula was connected to a volume-limited ventilator (Model SN-480-7, Shinano Manufacturing Co., Ltd., Tokyo, Japan) providing artificial ventilation at a supply rate of 4 ml/stroke and 70 strokes/min. Another catheter was inserted into the jugular vein to secure the route for drug administration. After confirming a stable ventilation pressure, 1 vol% WellSolve (0.5 ml/kg) was injected into each animal via the catheter inserted into the jugular vein. Approximately 1 min and 50 s later, bolus doses of physiological saline or S-hexyl GSH (15 mg/kg) and LTC₄ (15 μ g/kg) were intravenously injected to each guinea pig. Five minutes after administration of LTC₄, the chest was opened, and a sonde was inserted into the pulmonary artery from the right ventricle. The lung was then perfused with 50 ml of ice-cold perfusate (physiological saline containing 0.01 w/v% sodium citrate, 45 mM L-serine, 45 mM boric acid, 5 mM L-cysteine, 10 μ M indomethacin, and 10 μ M zileuton) and simultaneously a small incision was made on the left atrium. The lung subjected to perfusion was isolated together with the trachea and the bronchial cannula, and the lung lobes were separated to measure their wet weights. The isolated lung lobes were next homogenized in 4 ml of ice-cold 99.5 vol% ethanol using a polytron homogenizer (lung homogenate) and stored at -80 °C until quantification of CysLTs.

To create calibration curves for quantification of CysLTs, an aliquot (0.18 ml) of the lung homogenate was allowed to stand overnight at 60 °C in the presence of 1 M sodium hydroxide. Protein concentration in the lung homogenate was spectrophotometrically (Spectromax190, Molecular Devices, Tokyo, Japan) determined using the BCA[™] Protein Assay Kit (Thermo Fisher Scientific Inc, MA, USA) in accordance with the manufacturer's instructions. Bovine serum albumin was used as standard for the calibration curve.

2.6. Measurement of CysLTs concentrations in lung tissue

The lung homogenates were centrifuged at 1870g for 10 min at 4 °C (Hitachi Himac centrifuge, Rotor RT3S3, Tokyo, Japan), and the supernatants were collected (lung samples). A 50 µl portion of a standard solution (mixture of LTC₄, LTD₄, and LTE₄) for the calibration curve (0.6 to 600 ng/ml) was then added to 1 ml of each lung sample. A 50 µl portion of an internal standard (IS mixture; a mixture of LTC₄-d5, LTD₄-d5, and LTE₄-d5) was added to 1 ml of each lung sample for measurement and for the calibration curve. The lung samples were next evaporated to dryness by vacuum centrifuge (non-heated). The resultant residues were dissolved in 2 ml of distilled water and loaded on a solid phase extraction column (Oasis MAX cartridge 3 cc/60 mg 30 µm; Waters Corporation, MA, USA) pre-conditioned with 2 ml of methanol and 2 ml of distilled water. After washing the column with 2 ml of distilled water followed by 2 ml of 1 vol% aqueous ammonia and 2 ml of methanol, CysLTs were eluted with 2 ml of methanol containing 2 vol% formic acid. The eluate was evaporated to dryness by vacuum centrifuge and the residues were re-dissolved in 150 µl of a mixture of mobile phase A (0.2 vol% formic acid) and B (acetonitrile containing 0.2 vol% formic acid) (75/25). Individual levels of CysLTs in the samples were determined by LC/MS/MS (HPLC system: Prominence UFLC, Shimadzu, Kyoto, Japan; MS/MS: API 5000, AB Sciex, MA, USA). Concentrations of CysLTs in lung tissues were expressed in ng/g protein.

2.7. Measurement of bronchoconstriction

Bronchoconstriction was measured in accordance with the method described by Konzett and Rössler using ventilation pressure as an indicator (Konzett and Rössler, 1940). Briefly, guinea pigs were anesthetized with pentobarbital sodium (75 mg/kg, i.p.), and the trachea was cannulated with a polyethylene cannula that provided artificial ventilation as described in Section 2.5. Ventilation pressure was measured via a pneumotachometer (M·I·P·S Co., Ltd., Osaka, Japan) connected to a side port of the tracheal cannula, using a WinPULMOS-III system (Version 3.6, M·I·P·S Co., Ltd., Osaka, Japan). Compound A (1 mg/kg) and S-hexyl GSH (0, 15, 30, 60 mg/kg) were injected 1 min and 10 s prior to LTC₄ (15 µg/kg) injection, respectively, via a catheter secured in the jugular vein. Ventilation pressure was measured for at least 30 min after LTC₄ injection. The trachea was then completely blocked to obtain maximum ventilation pressure. After measurement of maximum ventilation pressure, the mechanical ventilation was stopped, and the animal was euthanized. The area under the percentage bronchoconstriction curve from 0 to 30 min after LTC₄ injection (AUC) was calculated by the trapezoidal method, and the mean percentage bronchoconstriction (%) was calculated by dividing AUC by measurement time.

2.8. Measurement of airway vascular permeability

Immediately after intravenous injection of FITC-BSA (2 mg/kg), the guinea pigs intravenously received S-hexyl GSH (60 mg/kg) and LTC₄ (2 µg/kg). Fifteen minutes after administration of LTC₄, pentobarbital sodium (Somnopentyl®: 19.4 mg/animal) was intravenously administered, and the animals were exsanguinated by cutting the femoral artery and vein followed by the abdominal aorta and vena cava. Thoracotomy was then performed, and the trachea was cannulated. The whole lung was gently lavaged 5 times with 10 ml of ice-cold physiological saline containing 3.8 mg/ml sodium citrate acid via the tracheal cannula to collect the bronchoalveolar lavage fluid (BALF), which was centrifuged at 1870g for 5 min at 4 °C (Hitachi Himac Centrifuge, Rotor RPRS3, Tokyo, Japan). Two-hundred micro-liter of the resultant supernatant was then transferred to a 96-well microplate (Corning Incorporated) under protection from light. Fluorescence intensity (Ex: 485 nm/Em: 538 nm) was measured for each supernatant using a microplate reader (Fmax, Nihon Molecular Devices Corporation, Tokyo, Japan) and its bundled software (SOFTmax PRO version 1.3.1f, Nihon Molecular Devices Corporation).

2.9. Statistical analysis

Results are expressed as the mean ± S.E.M., except for EC₅₀ and IC₅₀ values, and the maximum response in calcium signal. EC₅₀ and IC₅₀ values, and the maximum response for CysLT-stimulated intracellular calcium responses were estimated by a 4-parameter logistic model using GraphPad Prism 5.01 (GraphPad Software, Inc, CA, USA). Data were statistically analyzed using *t*-test for comparison between 2 groups with SAS 9.1.3 Service Pack 4 (SAS Institute Japan, Tokyo, Japan) and the linked system EXSAS Version 7.5.2 (CAC EXICARE Corporation, Tokyo, Japan). Significance level was set at 0.05.

3. Results

3.1. Calcium response assay

LTC₄, LTD₄ and LTE₄ did not induce significant intracellular calcium response in CHO-K1 cells (data not shown). The order of potency at the CysLT₁ receptor was LTD₄ > LTE₄ > LTC₄ with EC₅₀ values of 2.27, 260 and 355 nM, respectively. On the other hand, the order of potency at the CysLT₂ receptor was LTC₄ > LTD₄ > LTE₄ with EC₅₀ values of 0.649, 25.0 and 384 nM, respectively. Unlike at the CysLT₁ receptor, maximum calcium signal in response to LTC₄ was significantly higher than that in response to LTD₄ or LTE₄, suggesting that LTC₄ preferentially transduce receptor-associated signal at the CysLT₂ receptor (Fig. 1 and Table 1).

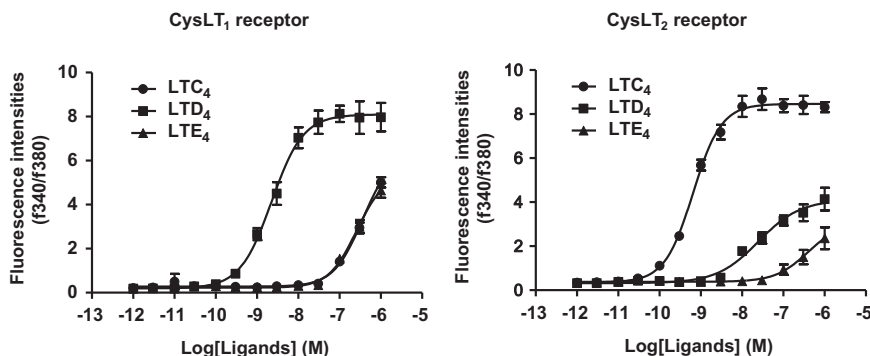


Fig. 1. Intracellular calcium response stimulated by LTC₄, LTD₄ and LTE₄ in CHO-K1 cells stably expressing guinea pig CysLT₁ and CysLT₂ receptors. Fura-2-AM-loaded CHO-K1 cells stably expressing guinea pig CysLT₁ or CysLT₂ receptor were stimulated with LTC₄, LTD₄ or LTE₄ and subsequently irradiated at 2 excitation wavelengths (340 and 380 nm) to monitor changes in fluorescence intensity at 500 nm. Maximum change in the ratio of fluorescence intensity (f340/f380) following the addition of each CysLT was determined. Each plot shows the mean ± S.E.M. for 3 independent experiments.

Montelukast concentration-dependently inhibited LTD₄-stimulated calcium response in CysLT₁ receptor-expressing cells with an IC₅₀ value of 1.6 nM. This inhibition was 3 orders of magnitude more potent than that of the CysLT₂ receptor (IC₅₀ value of 1800 nM). In sharp contrast to montelukast, Compound A preferentially inhibited CysLT₂ receptor-stimulated calcium signal with an IC₅₀ value of 0.64 nM compared to 2600 nM at the CysLT₁ receptor (Table 2).

3.2. Concentrations of CysLTs in lung tissues following LTC₄ administration

The amounts of CysLTs in lung tissues 5 min after intravenous injection of LTC₄ were determined in guinea pigs treated or non-treated with S-hexyl GSH. In the absence of S-hexyl GSH, the predominant CysLT was LTD₄ (724 ng/g protein) compared to LTE₄ (73 ng/g protein) and LTC₄ (9 ng/g protein), suggesting rapid metabolism of LTC₄ in *in vivo*. In contrast, LTC₄ (669 ng/g protein) was the most abundant CysLT in the lung tissues of S-hexyl GSH-treated animals, with LTD₄ (285 ng/g protein) being approximately at a concentration half that of LTC₄. Absolute concentrations of LTC₄ in the lungs of S-hexyl GSH-treated animals were significantly higher than those in the non-treated animals. Contrary to LTC₄, the concentrations of LTD₄ and LTE₄ in the S-hexyl GSH-treated animals were significantly lower than those in the non-treated animals (Fig. 2). LTC₄/(LTD₄+LTE₄) ratio, an indicator of LTC₄ metabolism to LTD₄, in the lungs of S-hexyl GSH-treated animals was much higher than that in the non-treated animals (1.64 vs. 0.01).

3.3. LTC₄-induced bronchoconstriction

In the absence of S-hexyl GSH, LTC₄ (15 µg/kg) elicited bronchoconstriction that was abrogated by montelukast, but not by Compound A. However, LTC₄ at the same dose caused a more pronounced bronchoconstriction when the animals were treated with S-hexyl GSH at 15 mg/kg or more. This LTC₄-induced bronchoconstriction was partially inhibited by either montelukast or Compound A. At the highest dose of S-hexyl GSH (60 mg/kg), the inhibitory effect of montelukast on LTC₄-induced bronchoconstriction was completely abolished (Fig. 3). On the other hand, Compound A inhibition of LTC₄-induced bronchoconstriction increased with increasing dose of S-hexyl GSH. Combination of montelukast and Compound A completely blocked this response at every evaluation point in S-hexyl GSH (15 mg/kg)-treated animals (Fig. 4).

3.4. LTC₄-induced airway vascular hyperpermeability

Intravenous injection of LTC₄ induced airway vascular hyperpermeability in both S-hexyl GSH-treated and non-treated guinea pigs. Montelukast completely inhibited this hyperpermeability in the non-treated animals (data not shown), but failed to do so in S-hexyl GSH-treated animals. On the other hand, Compound A inhibited

LTC₄-induced hyperpermeability to a level comparable to that of normal or S-hexyl GSH (60 mg/kg)-treated guinea pigs that received a combination of montelukast and Compound A (Fig. 5).

4. Discussion

Due to species difference in the affinity of LTD₄ for CysLT₂ receptors between human and the guinea pig, studying the functional role of CysLT₂ receptors in asthma using guinea pig models has been very difficult. By inhibiting LTC₄ metabolism using S-hexyl GSH, we succeeded in developing a guinea pig model of asthma where bronchoconstriction and airway vascular permeability are mediated not only via CysLT₁ receptors, but also via CysLT₂ receptors. Inhibition of LTC₄ metabolism resulted in an increase of this ligand concentration in guinea pig lung tissue, leading to a CysLT₂ receptor-dependent

Table 2

IC₅₀ values of montelukast and Compound A for inhibition of CysLT-induced intracellular calcium response.

Guinea pig receptor	IC ₅₀ (nM)	
	Montelukast	Compound A
CysLT ₁ receptor	1.6 (1.1–2.2)	2600 (2100–3100)
CysLT ₂ receptor	1800 (1600–2000)	0.64 (0.53–0.76)

Fura 2-AM-loaded CHO-K1 cells expressing guinea pig CysLT₁ or CysLT₂ receptor were stimulated with 10 nM LTD₄ for the CysLT₁ receptor, or 3 nM LTC₄ for the CysLT₂ receptor. IC₅₀ values were estimated by the 4-parameter logistic model using data obtained from 5 separate experiments. Parameters top and bottom were constrained by 100 and 0, respectively. Values indicated in parentheses represent 95% confidence intervals of the IC₅₀ value.

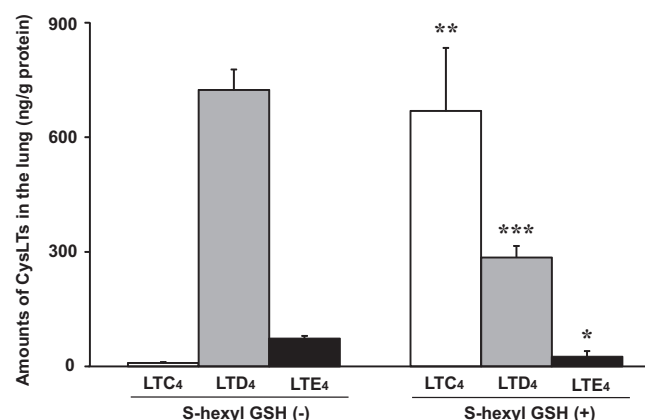


Fig. 2. Concentrations of LTC₄, LTD₄ and LTE₄ in guinea pigs lung tissues treated or non-treated with S-hexyl GSH. Animals were intravenously injected with LTC₄ (15 µg/kg), and lung tissues were isolated 5 min later. S-hexyl GSH (15 mg/kg) or physiological saline was intravenously administered approximately 10 s before LTC₄ injection. Each column represents the mean ± S.E.M. for 6 animals. *, *P* < 0.05, **, *P* < 0.01, ***, *P* < 0.001: *t*-test vs. corresponding CysLT in S-hexyl GSH(-) group.

Table 1

Potency and efficacy of LTC₄, LTD₄ and LTE₄ for CysLT-induced intracellular calcium response in CHO-K1 cells stably expressing guinea pig CysLT₁ receptors and CysLT₂ receptors.

Guinea pig receptor	EC ₅₀ (nM)			Maximal response (f340/f380)		
	LTC ₄	LTD ₄	LTE ₄	LTC ₄	LTD ₄	LTE ₄
CysLT ₁ receptor	355 (216–582)	2.27 (1.72–2.98)	260 (179–375)	6.31 (4.87–7.75)	8.10 (7.68–8.51)	5.41 (4.52–6.29)
CysLT ₂ receptor	0.649 (0.541–0.779)	25.0 (14.2–44.2)	384 (56.8–2600)	8.46 (8.21–8.71)	4.11 (3.55–4.66)	3.03 (0.727–5.34)

CHO-K1 cells expressing guinea pig CysLT₁ or CysLT₂ receptor were stimulated with LTC₄, LTD₄ or LTE₄ to evaluate each receptor response using increase in intracellular calcium as an indicator. EC₅₀ (nM) value and maximum calcium response, and their 95% confidence intervals (in parentheses) were estimated by the 4-parameter logistic model using data from 3 separate experiments.

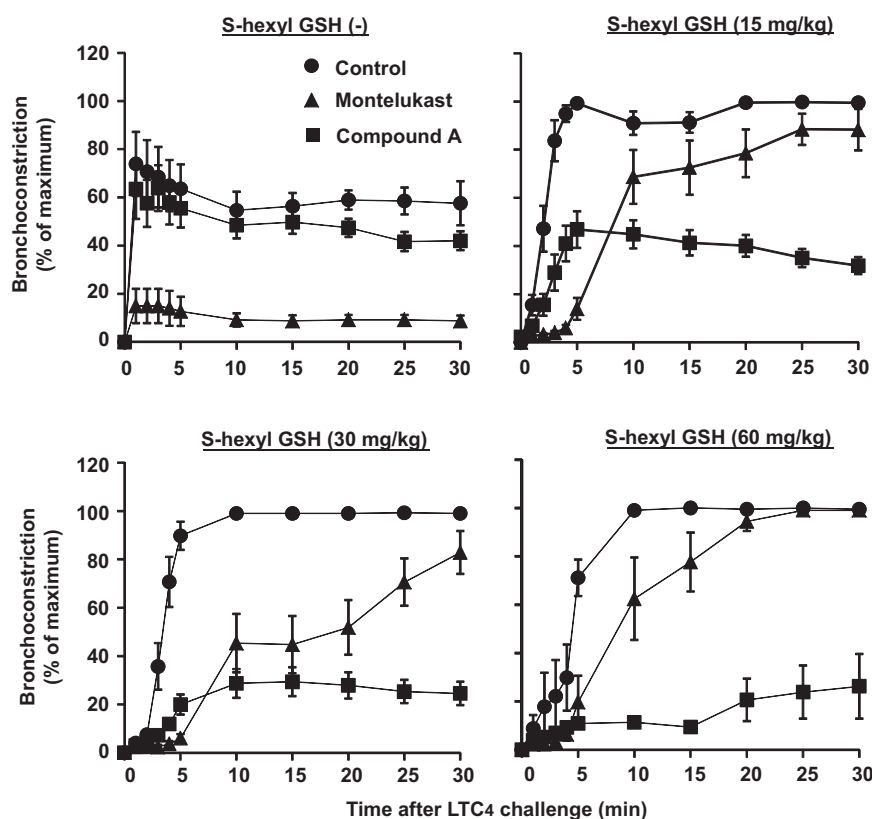


Fig. 3. Effects of montelukast and Compound A on LTC₄-induced bronchoconstriction in guinea pigs treated with varying doses of S-hexyl GSH. Animals treated with S-hexyl GSH (0, 15, 30, and 60 mg/kg) were challenged with LTC₄ (15 µg/kg) under anesthesia. Montelukast was orally administered 24 h before LTC₄. Compound A was intravenously administered approximately 1 min before LTC₄. Each column represents the mean ± S.E.M. for 6–11 animals.

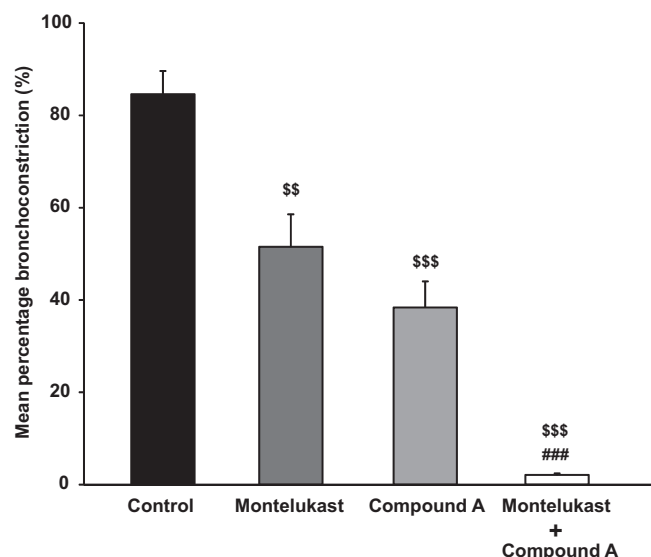


Fig. 4. Effects of montelukast, Compound A, and combination of montelukast and Compound A on LTC₄-induced bronchoconstriction in S-hexyl GSH-treated guinea pigs. Animals treated with S-hexyl GSH (15 mg/kg) were challenged intravenously with LTC₄ (15 µg/kg) under anesthesia. Montelukast was orally administered 24 h before LTC₄. Compound A was intravenously administered approximately 1 min before LTC₄. Each column represents the mean ± S.E.M. for 10 animals. \$, $P < 0.01$; \$, $P < 0.001$; t-test vs. control group, ###, $P < 0.001$; t-test vs. montelukast group.

biological response as evidenced by Compound A (a specific CysLT₂ receptor antagonist) inhibition of LTC₄-induced bronchoconstriction. The developed model may therefore be useful to elucidate the functional role of CysLT₂ receptors in various diseases including asthma.

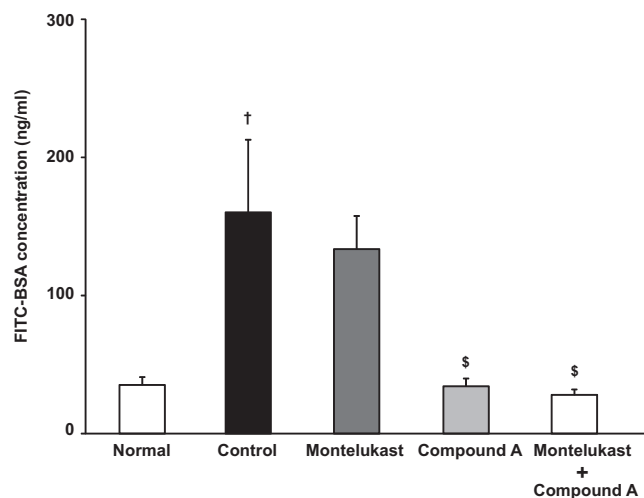


Fig. 5. Effect of montelukast, Compound A and combination of montelukast and Compound A on LTC₄-induced airway vascular hyperpermeability in S-hexyl GSH-treated guinea pigs. Immediately after FITC-BSA injection, S-hexyl GSH (60 mg/kg) and LTC₄ (2 µg/kg) were intravenously administered. Fifteen minutes later, BALF was collected, and FITC-BSA level in the BALF supernatant was determined. Montelukast was orally administered 24 h before LTC₄. Compound A was intravenously administered just before LTC₄. Each column represents the mean ± S.E.M. for 18 animals. †, $P < 0.05$; t-test vs. normal group, \$, $P < 0.05$; t-test vs. control group.

It has been shown that LTC₄ is more than a hundred times more potent than LTD₄ in activating calcium mobilization through CysLT₂ receptors in guinea pigs (Ito et al., 2008). This finding indicates that the guinea pig CysLT₂ receptor is preferentially activated by LTC₄. In the present study, we confirmed that LTC₄ selectively stimulates guinea pig CysLT₂, while LTD₄ readily binds

to CysLT₁ receptors (Table 1). On the other hand, in humans, CysLTs affinity for CysLT₁ receptors is reported to be in the order of LTD₄ > LTC₄ ≈ LTE₄, while that for CysLT₂ receptors is in the order of LTC₄ = LTD₄ > LTE₄ (Lynch et al., 1999; Heise et al., 2000). Therefore, in humans, LTD₄ is the most potent natural agonist for both CysLT₁ and CysLT₂ receptors. In guinea pigs, on the other hand, LTC₄ is quickly metabolized to LTD₄, making it difficult to induce CysLT₂ receptor-mediated biological responses.

Considering that LTC₄ is rapidly transformed to LTD₄ by metabolizing enzymes, such as γ -GTP (Orning and Hammarström, 1980; Snyder et al., 1984) and gamma-glutamyl leukotrienase (Lieberman et al., 1999; Han et al., 2002), we measured CysLTs levels in guinea pigs plasma and lung tissue after LTC₄ administration. As expected, LTC₄ was hardly detected in the plasma and lung tissue, instead high levels of LTD₄ were measured (Fig. 2). In fact, we confirmed that intravenous administration of LTC₄ induces bronchoconstriction in normal guinea pigs and that this bronchoconstriction is fully abrogated by the CysLT₁ selective antagonist, montelukast, but not by the CysLT₂ receptor antagonist Compound A (Fig. 3). These results indicate that exogenously administered LTC₄ is rapidly metabolized to LTD₄ and the LTD₄ induces bronchoconstriction via a CysLT₁ receptor-dependent pathway in normal guinea pigs.

To suppress LTC₄ metabolism in guinea pig plasma and lung tissue, we focused on GSH derivatives, which are synthetic substrates of γ -GTP known as γ -GTP inhibitors (Bäck et al., 2001). As anticipated, the concentration of LTC₄ and the ratio of LTC₄/(LTD₄ + LTE₄) in the lung tissue dramatically increased in S-hexyl GSH-treated animals. In the presence of S-hexyl GSH, intravenous administration of LTC₄ induced bronchoconstriction to a degree much higher than that the untreated animals. The inhibitory effect of montelukast on LTC₄-induced bronchoconstriction subsided with increasing doses of S-hexyl GSH, although that of Compound A became more potent, suggesting that the contribution of CysLT₁ and CysLT₂ receptors to bronchoconstriction changed depending on LTC₄ concentration in the body (Fig. 3). In animals treated with 15 mg of S-hexyl GSH, dual blockage of CysLT₁ and CysLT₂ by combination treatment with montelukast and Compound A abolished LTC₄-induced bronchoconstriction, indicating involvement of both receptors in this condition. Thus, inhibition of LTC₄ metabolism allowed the involvement of both CysLT₁ and CysLT₂ receptors in LTC₄-induced biological response in guinea pigs. As LTD₄ is known to activate both human CysLT₁ and CysLT₂ receptors, it would be interesting to see if both CysLT₁ and CysLT₂ receptors are involved in human bronchoconstriction. It has been shown that a CysLT₁ receptor antagonist does not completely inhibit tracheal contraction induced by high concentrations of LTD₄ and that residual contraction to LTD₄ is inhibited by the CysLT₁/LT₂ receptor antagonist BAY u9773 (Bäck et al., 2001; Tudhope et al., 1994).

In addition to bronchoconstriction, CysLTs also induce potent airway vascular hyperpermeability (Nakagawa et al., 1992). As is the case with bronchoconstriction, intravenous administration of LTC₄ to guinea pigs induced airway vascular hyperpermeability, which was fully abrogated by the CysLT₁ receptor antagonist montelukast. However, our study shows that in the presence of 60 mg/kg S-hexyl GSH, LTC₄-induced airway vascular hyperpermeability was not inhibited by montelukast, but was almost completely abolished by Compound A. These results clearly demonstrate that LTC₄ induced airway vascular hyperpermeability via both CysLT₁ and CysLT₂ receptors.

Interestingly, LTC₄-induced bronchoconstriction was rather aggravated by S-hexyl GSH. We have previously found in an *in vitro* study that LTC₄ in the presence of a γ -GTP inhibitor elicits more potent contraction of guinea pig trachea than LTD₄ or LTC₄ in the absence of the γ -GTP inhibitor (Obata et al., 1992). Additionally, in the S-hexyl GSH (15 mg/kg)-treated guinea pigs, montelukast seemed to suppress

the early phase of LTC₄-induced bronchoconstriction, while Compound A inhibited the later phase. It is therefore suggested that CysLT₂ receptor-mediated bronchoconstriction is more severe and sustained than that mediated by the CysLT₁ receptor. In fact, we have already confirmed that inhaled LTC₄ causes more severe and sustained bronchospasm in conscious guinea pigs treated with S-hexyl GSH than inhaled LTD₄ (data not shown). However, the difference between the effect of LTC₄ and that of LTD₄ cannot be explained by receptor density and calcium signal response due to the following reasons. First, both CysLT₂ and CysLT₁ receptors are expressed in the lung of guinea pigs. Second, the expression level of mRNA for the guinea pig CysLT₂ receptor is even lower than that of the CysLT₁ receptor in the lung tissue (data not shown). Finally, there appeared to be no significant difference in the maximum response of calcium signal between the two receptors. Further studies are needed to address the functional difference between guinea pig CysLT₁ and CysLT₂ receptors. In addition, the physiological function of the CysLT₂ receptor is not fully clear, although CysLT₂ receptors are expressed on many cell types including eosinophil (Mita et al., 2001), monocyte/macrophage (Figueroa et al., 2003), mast cell (Mellor et al., 2003), airway smooth muscle cell (Heise et al., 2000), platelet (Hasegawa et al., 2010), cardiac Purkinje cell (Heise et al., 2000) and endothelial cell (Sjöström et al., 2003). The animal model constructed in this study using S-hexyl GSH may be useful for studying the role of the CysLT₂ receptor not only in bronchoconstriction and airway vascular hyperpermeability, but also in other conditions.

Currently, selective CysLT₁ receptor antagonists as well as inhaled corticosteroids are widely used in the treatment asthma (Montuschi and Peters-Golden, 2010). However, a sizable proportion of patients do not respond fully to these therapies. As demonstrated in this study, CysLT₂ receptors, like CysLT₁ receptors, can mediate asthmatic response, suggesting that dual antagonist for CysLT₁/LT₂ receptors antagonists can be more useful for the treatment of asthma than specific CysLT₁ receptor antagonists. The developed model may therefore become a powerful tool for screening and characterization of such new anti-asthmatic agents.

In conclusion, we have established a novel guinea pig model of asthma mediated via the CysLT₂ receptor using S-hexyl GSH. This model may be useful to study the functional role of CysLT₂ receptors in various diseases, including asthma.

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